nature medicine

Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells

Alexandra Aicher[1,3], Christopher Heeschen[1,3], Christiane Mildner-Rihm[1], Carmen Urbich[1], Christian Ihling[2], Katja Technau-Ihling[2], Andreas M Zeiher[1] & Stefanie Dimmeler[1]

Endothelial nitric oxide synthase (eNOS) is essential for neovascularization. Here we show that the impaired neovascularization in mice lacking eNOS is related to a defect in progenitor cell mobilization. Mice deficient in eNOS (*Nos3*$^{-/-}$) show reduced vascular endothelial growth factor (VEGF)-induced mobilization of endothelial progenitor cells (EPCs) and increased mortality after myelosuppression. Intravenous infusion of wild-type progenitor cells, but not bone marrow transplantation, rescued the defective neovascularization of *Nos3*$^{-/-}$ mice in a model of hind-limb ischemia, suggesting that progenitor mobilization from the bone marrow is impaired in *Nos3*$^{-/-}$ mice. Mechanistically, matrix metalloproteinase-9 (MMP-9), which is required for stem cell mobilization, was reduced in the bone marrow of *Nos3*$^{-/-}$ mice. These findings indicate that eNOS expressed by bone marrow stromal cells influences recruitment of stem and progenitor cells. This may contribute to impaired regeneration processes in ischemic heart disease patients, who are characterized by a reduced systemic NO bioactivity.

Improved neovascularization is an important therapeutic goal after myocardial infarction or limb ischemia[1]. In recent years, our understanding of the processes responsible for the formation of new blood vessels after tissue ischemia has changed. Vascularization of ischemic tissue in adults was once thought to be restricted to migration and proliferation of mature endothelial cells, a process termed 'angiogenesis'[2]. However, increasing evidence suggests that circulating EPCs home to sites of ischemia and contribute to the formation of new blood vessels[3–5]. In analogy to the embryonic development of blood vessels from primitive endothelial progenitors (angioblasts), this process is referred to as 'vasculogenesis'[6,7]. The importance of circulating progenitor cells is demonstrated by the fact that genetic inhibition of their recruitment inhibits tumor angiogenesis[8], whereas intravenous infusion of bone marrow–derived progenitor cells augments neovascularization[9]. EPCs can be mobilized from the bone marrow into the circulation by VEGF or stromal cell–derived factor-1 (SDF-1)[10–12]. Maintenance and mobilization of stem cells in the bone marrow is determined by the local microenvironment, which consists of fibroblasts and endothelial cells[13–15]. VEGF- and SDF-1-induced progenitor cell mobilization is dependent on local secretion of MMP-9 by the hematopoietic and stromal compartments of the bone marrow, which results in release of soluble Kit ligand (KitL; also known as stem cell factor)[11]. This permits transfer of endothelial and hematopoietic progenitor cells from the quiescent to the proliferative niche[11].

Nos3$^{-/-}$ mice, which are deficient in eNOS, are characterized by impaired ischemia-induced neovascularization[16]. Mobilization of EPCs from the bone marrow into the circulation is required for postnatal neovascularization[4]. Here we show that mice lacking eNOS have defective hematopoietic recovery and progenitor cell mobilization, resulting in increased mortality after myelosuppression and reduced VEGF-induced mobilization of EPCs. Intravenous infusion, but not bone marrow transplantation, of wild-type stem and progenitor cells rescued the impaired neovascularization of *Nos3*$^{-/-}$ mice in a model of hind-limb ischemia.

RESULTS

Mobilization of progenitor cells in *Nos3*$^{-/-}$ mice

Basal numbers of EPCs did not significantly differ between *Nos3*$^{-/-}$ and wild-type mice (**Fig. 1a**). However, whereas the number of peripheral EPCs significantly ($P < 0.05$) increased after VEGF mobilization in wild-type mice, *Nos3*$^{-/-}$ mice did not mobilize EPCs in response to VEGF (**Fig. 1a**). These data were confirmed by directly measuring the number of $CD34^{+}Flk\text{-}1^{+}$ cells by fluorescence-activated cell sorting (FACS) analysis (data not shown). To assess whether the reduced mobilization capacity in *Nos3*$^{-/-}$ mice consequently affects hematopoietic reconstitution after myelosuppression, *Nos3*$^{-/-}$ mice were treated with 5-fluorouracil (5-FU), which allows for the study of factors that regulate recruitment of stem cells during hematopoietic recovery[11]. *Nos3*$^{-/-}$ mice had significantly ($P < 0.02$) increased mortality compared with wild-type mice (**Fig. 1b**). The recovery of the white blood cell count was significantly delayed in *Nos3*$^{-/-}$ mice ($P = 0.01$; **Fig. 1c** and **Supplementary Fig. 1** online). In line with these findings, we observed that the increase in cellularity

[1]Molecular Cardiology, Department of Internal Medicine IV, University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. [2]Department of Pathology, University of Freiburg, Albertstrasse 19, 79104 Freiburg, Germany. [3]These authors contributed equally to this work. Correspondence should be addressed to S.D. (Dimmeler@em.uni-frankfurt.de).

Published online 12 October 2003; doi:10.1038/nm948

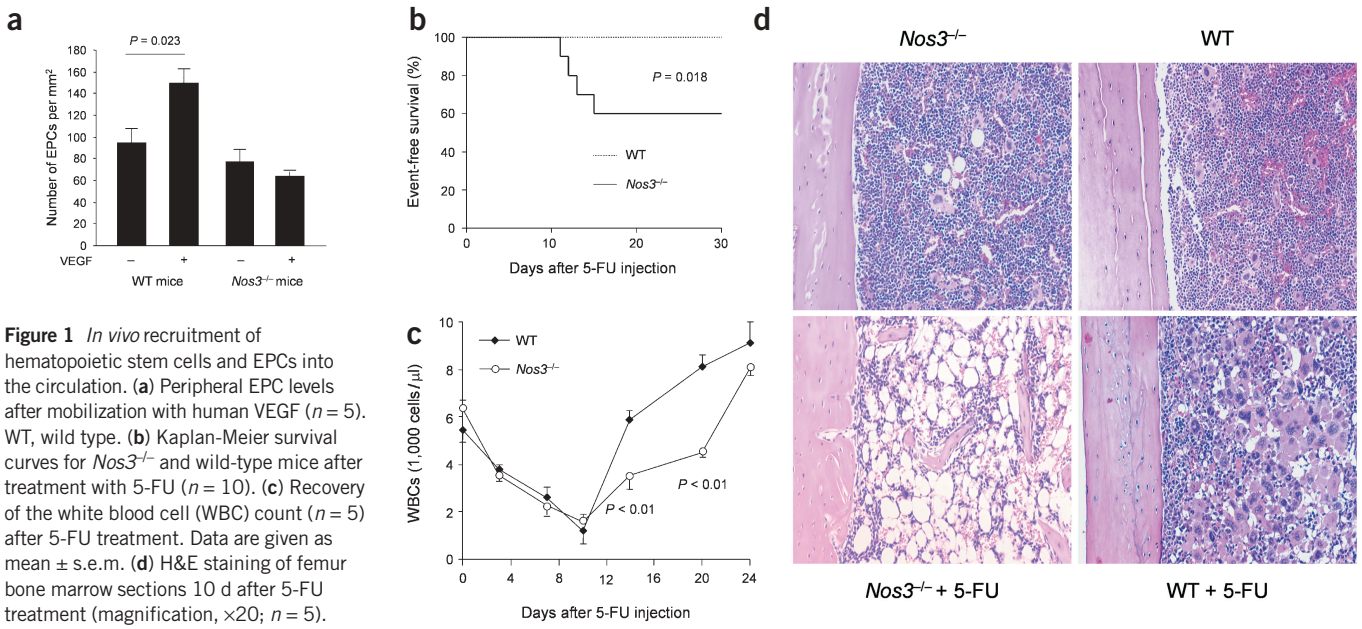

Figure 1 *In vivo* recruitment of hematopoietic stem cells and EPCs into the circulation. (**a**) Peripheral EPC levels after mobilization with human VEGF ($n = 5$). WT, wild type. (**b**) Kaplan-Meier survival curves for *Nos3*$^{-/-}$ and wild-type mice after treatment with 5-FU ($n = 10$). (**c**) Recovery of the white blood cell (WBC) count ($n = 5$) after 5-FU treatment. Data are given as mean ± s.e.m. (**d**) H&E staining of femur bone marrow sections 10 d after 5-FU treatment (magnification, ×20; $n = 5$).

and hematopoietic cell clusters detected in histological bone marrow sections of wild-type mice, 6–10 d after 5-FU treatment, was incomplete in *Nos3*$^{-/-}$ mice (**Fig. 1d**). These data indicate that eNOS is essential for mobilization and hematopoietic reconstitution.

Effect of progenitor cells on neovascularization

We used a hind-limb ischemia model to assess whether impaired mobilization of EPCs, which may contribute to the reduced neovascularization in *Nos3*$^{-/-}$ mice, can be counteracted by infusion of wild-type stem and progenitor cells. As previously reported[16], *Nos3*$^{-/-}$ mice showed significantly reduced limb perfusion 2 weeks after induction of ischemia, compared with wild-type mice ($P < 0.01$; **Fig. 2a**,**b**). The impaired limb perfusion was rescued by intravenous infusion of wild-type EPCs or isolated wild-type bone marrow cells (BMCs; sca-1$^+$Lin$^-$; **Fig. 2a**,**b**). Histological analysis of ischemic thigh muscles indicated that infusion of wild-type EPCs or BMCs substantially increased capillary density (**Fig. 2c**,**d**) and number of conductant vessels (**Fig. 2e**) in *Nos3*$^{-/-}$ mice. Physical incorporation of intravenously infused wild-type cells into endothelial structures was confirmed by immunostaining for CD31 or mouse pan–endothelial cell antigen-32 (MECA-32) (**Fig. 3**).

To investigate whether impaired stem cell–stromal cell interaction contributes to defective neovascularization in *Nos3*$^{-/-}$ mice, bone marrow cells depleted of stromal cells by CD45 magnetic bead selection were transplanted into lethally irradiated *Nos3*$^{-/-}$ mice. After completely reconstituting the bone marrow of *Nos3*$^{-/-}$ mice with wild-type stem cells, the mice were treated with VEGF for three consecutive days. VEGF-induced mobilization of white blood cells or EPCs was blunted in the *Nos3*$^{-/-}$ mice transplanted with wild-type bone marrow (**Fig. 4a**,**b**). In contrast, VEGF significantly ($P = 0.006$) increased peripheral white blood cell counts in wild-type mice transplanted with *Nos3*$^{-/-}$ bone marrow (**Fig. 4a**). Similarly, VEGF significantly increased c-Kit$^+$Sca-1$^+$ peripheral blood cells in wild-type mice transplanted with wild-type bone marrow (211 ± 43% increase; $P = 0.03$ compared with unstimulated mice; $n = 6$ per group), and in wild-type mice engrafted with *Nos3*$^{-/-}$ bone marrow (398 ± 91% increase; $P = 0.01$ versus unstimulated mice). In contrast, no significant increase was detected when VEGF was injected into *Nos3*$^{-/-}$ mice transplanted with wild-type bone marrow (141 ± 26%; $P = 0.186$).

We used a model of hind-limb ischemia to investigate the functional relevance of this impaired mobilization in *Nos3*$^{-/-}$ mice. In contrast to the improvement of neovascularization induced by intravenous infusion of wild-type progenitor cells (**Fig. 2**), bone marrow reconstitution with wild-type cells did not rescue the impaired neovascularization phenotype of *Nos3*$^{-/-}$ mice (**Fig. 4c**). Taken together, these data suggest that wild-type stem and progenitor cells are only effective when applied to the circulation, but cannot be mobilized from the bone marrow of *Nos3*$^{-/-}$ mice. The data also provide evidence that eNOS expression in the bone marrow is essential for mobilization of stem and progenitor cells.

Bypassing the mobilization of BMCs by intravenously infusing *Nos3*$^{-/-}$ cells instead of wild-type cells is insufficient to compensate for the impaired recovery of *Nos3*$^{-/-}$ mice after ischemia (**Fig. 2a–d**). Consistently, a significant ($P < 0.05$) impairment of neovascularization was observed when *Nos3*$^{-/-}$ BMCs were transplanted into wild-type mice (**Fig. 4c**), despite the effective mobilization of white blood cells by VEGF (**Fig. 4a**). These data suggest that eNOS is not only essential for mobilization of stem and progenitor cells from the bone marrow, but also determines their angiogenic capacity in ischemic tissue. The data also underscore the relative importance of bone marrow–derived cells for the angiogenic response to ischemia.

To obtain further insight into the impaired proangiogenic activity of *Nos3*$^{-/-}$ cells after intravenous application, we injected labeled wild-type or *Nos3*$^{-/-}$ cells into *Nos3*$^{-/-}$ mice and counted the cells that incorporated into the capillaries of the ischemic limb after 7 days. A large number of systemically injected wild-type cells incorporated into the microvessels of the ischemic limb and expressed endothelial marker proteins such as CD31 or MECA-32, whereas incorporation of *Nos3*$^{-/-}$ cells was significantly reduced ($P = 0.027$; **Fig. 3**). In addition, fewer Y-chromosome-positive cells were detected in capillaries when wild-type male BMCs were transplanted into female *Nos3*$^{-/-}$ recipients (**Fig. 4d**,**e**). Because of the

limited efficiency of Y-chromosome detection in histological sections (40%; ref. 17), the estimated percentage of capillaries incorporating bone marrow–derived cells after ischemia in our study was ~5% in wild-type mice previously transplanted with wild-type bone marrow. This figure was confirmed using wild-type mice transplanted with green fluorescent protein–expressing bone marrow. We found that 7 ± 11% of the vessels incorporated bone marrow–derived cells expressing endothelial marker proteins (**Supplementary Figs. 2–5** online). Taken together, these data suggest that wild-type cells are efficiently incorporated into vessels and express endothelial marker proteins, whereas eNOS-deficient cells are impeded by a marked reduction in homing and incorporation into microvessels.

Stromal and progenitor cell functions *in vitro*

Because our data indicate an impaired interaction between stem cells and stromal cells in the bone marrow of *Nos3*$^{-/-}$ mice, we analyzed the expression pattern of eNOS in different cellular components of bone marrow from wild-type mice. Whereas stromal cells strongly express eNOS, selected CD45^{+} bone marrow leukocytes showed only very faint eNOS expression (**Fig. 5a**). Additional subfractionation of cultivated stromal cells revealed that eNOS is predominantly expressed in MECA-32^{+} vascular cells (**Fig. 5a** and **Supplementary Fig. 6** online). The effect of eNOS on stem and progenitor cell function was further investigated by determining the proliferative and colony-forming activity of bone marrow stem cells. In the bone marrow of *Nos3* deficient mice, the numbers of proliferating bromodeoxyuridine (BrdU)-positive Lin^{-}Sca-1^{+} cells (**Fig. 5b,c**) and colony-forming units (**Fig. 5d**) were significantly reduced ($P < 0.05$). Because the bone marrow microenvironment created by stromal cells may influence the growth of stem and progenitor cells in the bone marrow, we isolated stromal cells from *Nos3*$^{-/-}$ or wild-type mice to generate feeder cells for functional long-term *in vitro* stem cell assays. We then cultured total bone marrow from wild-type mice on *Nos3*$^{-/-}$ or wild-type feeder cells to grow stem cells with repopulating potential and colony-forming capacity. When bone marrow mononuclear cells were grown on *Nos3*$^{-/-}$ feeders, the number of colony-forming units was significantly reduced ($P < 0.01$; **Fig. 5e**), indicating that stromal cell–derived NO is important for the culture of long-term stem

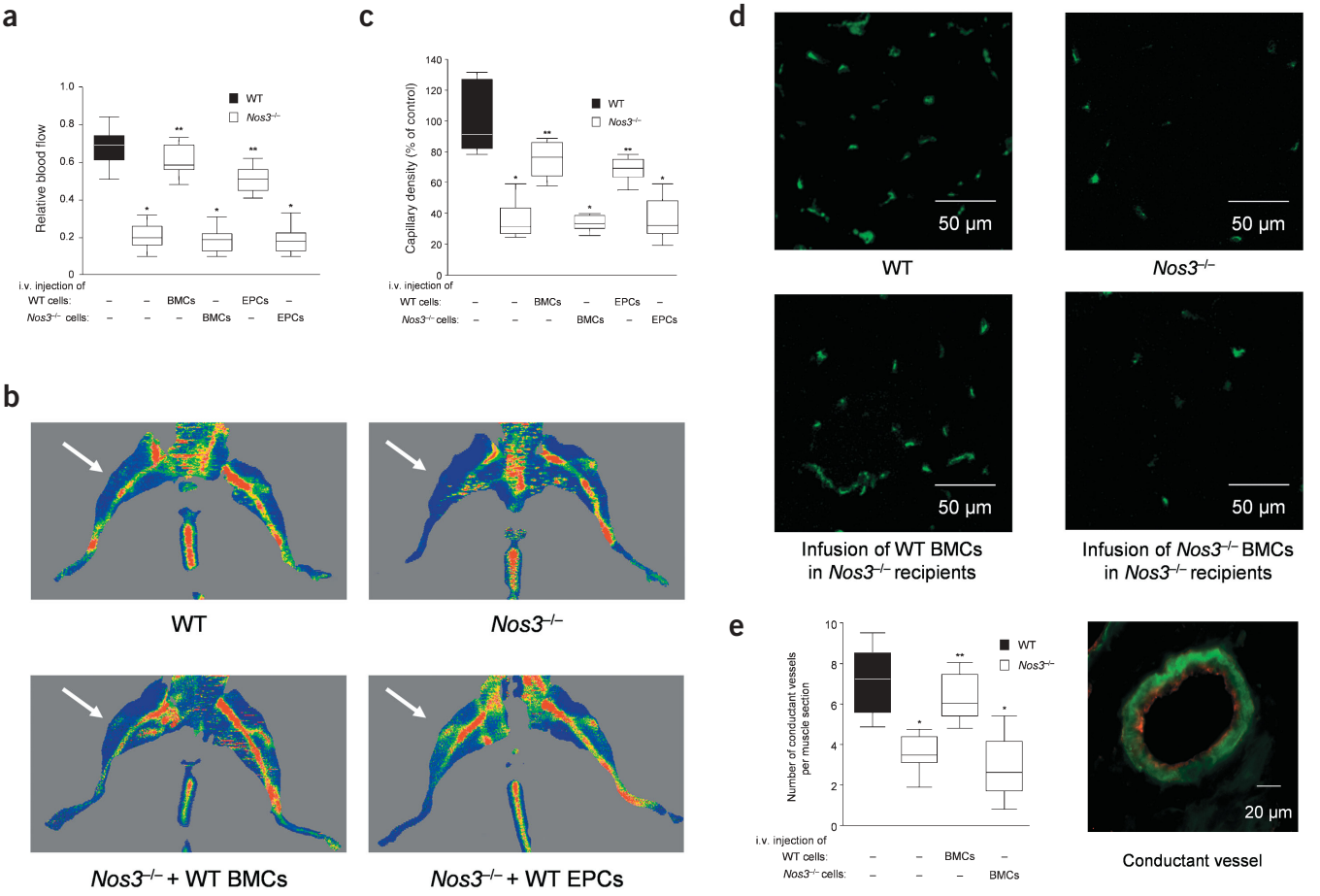

Figure 2 Rescue of impaired angiogenic phenotype in eNOS-deficient mice by intravenous (i.v.) injection of wild-type cells. (**a**) Laser Doppler–derived relative blood flow in *Nos3*$^{-/-}$ mice 14 d after induction of hind-limb ischemia and injection of wild-type or *Nos3*$^{-/-}$ cells (Sca-1^{+}Lin^{-} BMCs or *ex vivo*–cultured EPCs) ($n = 6$). Wild-type animals were used as a positive control. (**b**) Representative laser Doppler scans, with low or no perfusion displayed in dark blue and highest perfusion displayed in red. Arrows indicate ischemic leg. (**c**,**d**) Capillary density in wild-type and *Nos3*$^{-/-}$ mice relative to wild-type control animals ($n = 6$). Representative overview images are shown in **d**. (**e**) Left, numbers of conductant vessels in 4-mm^2 muscle sections from wild-type and *Nos3*$^{-/-}$ mice and wild-type control animals. Right, conductant vessels were identified by size (>20 µm) and α-actin immunostaining. **a**,**c**,**e**, *, $P < 0.01$ compared with wild-type; **, $P < 0.01$ compared with untreated *Nos3*$^{-/-}$ mice.

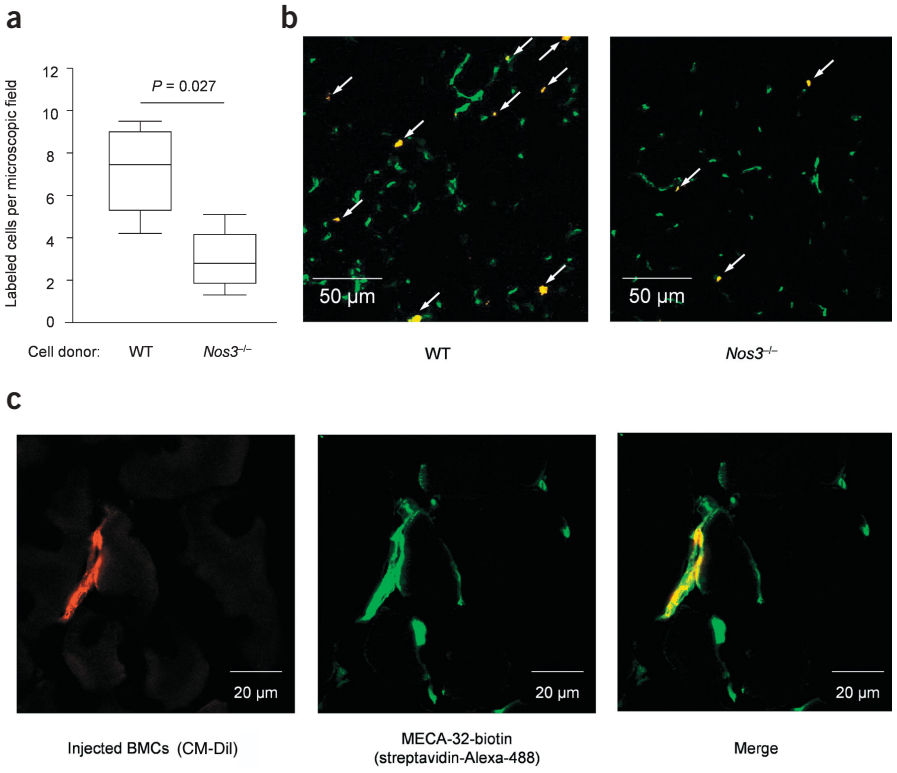

Figure 3 Incorporation of infused CellTracker-labeled BMCs into the vascular structures of limb muscles of *Nos3*$^{-/-}$ mice on day 7 after induction of ischemia. (**a**) Infused *Nos3*$^{-/-}$ BMCs showed a significantly lower incorporation rate compared with wild-type (WT) BMCs ($n = 4$). (**b**) Representative overview pictures of muscle sections. Arrows indicate incorporated BMCs (yellow) that are positive for CellTracker CM-DiI (red) and the endothelial marker CD31 (green). (**c**) High-power magnification of a vessel with incorporated wild-type BMCs that are positive for CellTracker CM-DiI (red) and the endothelial marker MECA-32 (green).

cells. Although eNOS was only weakly expressed in CD45$^+$ bone marrow leukocytes, we observed a significantly ($P < 0.01$) reduced number of colony-forming units when *Nos3*$^{-/-}$ bone marrow cells were grown on wild-type stromal feeders (**Fig. 5a,e**). This impairment of *Nos3*-deficient bone marrow cells was not related to increased apoptosis *in vitro* (data not shown). These results suggest that stem and progenitor cells derived from *Nos3*$^{-/-}$ mice are already partially impaired when grown *ex vivo* on wild-type feeder layers. These data are in line with our *in vivo* observation that intravenous infusion of *Nos3*$^{-/-}$ BMCs into *Nos3*$^{-/-}$ mice did not rescue the impaired neovascularization.

Pro-MMP-9 levels and activity in *Nos3*$^{-/-}$ mice

Finally, we examined the potential mechanisms underlying the impaired functional activity and mobilization of stem and progenitor cells in *Nos3*$^{-/-}$ mice. The expression of classical stem cell regulatory factors such as the β_1 integrin CD29 (ref. 18), adhesion molecules such as CD44 (ref. 19) and the chemokine receptor CXCR4 (ref. 20) were not altered in bone marrow cells of *Nos3*$^{-/-}$ mice (**Fig. 6a**). MMP-9, which is essential for VEGF- and 5-FU-induced stem cell mobilization[11,21], is activated by NO[22]. Indeed, pro-MMP-9] was significantly ($P < 0.01$) reduced in bone marrow plasma from *Nos3*$^{-/-}$ mice (**Fig. 6b**). In addition, the bone marrow of

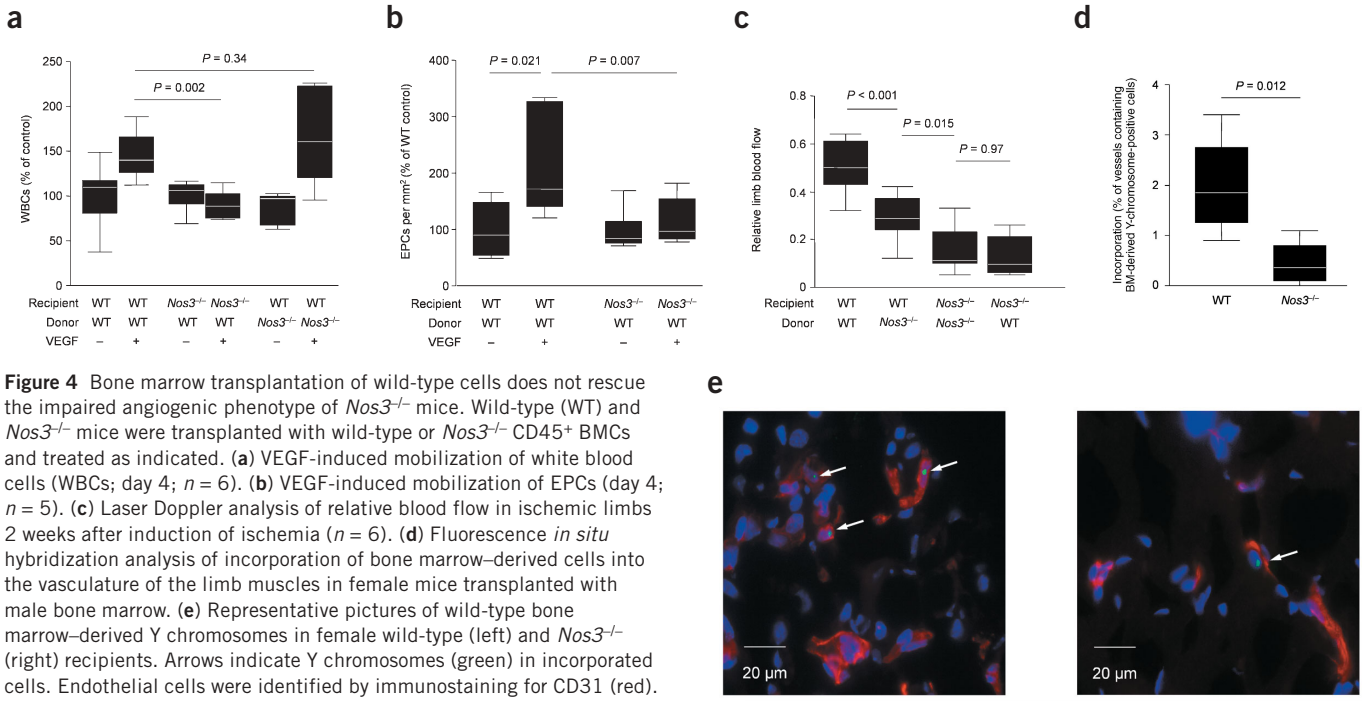

Figure 4 Bone marrow transplantation of wild-type cells does not rescue the impaired angiogenic phenotype of *Nos3*$^{-/-}$ mice. Wild-type (WT) and *Nos3*$^{-/-}$ mice were transplanted with wild-type or *Nos3*$^{-/-}$ CD45$^+$ BMCs and treated as indicated. (**a**) VEGF-induced mobilization of white blood cells (WBCs; day 4; $n = 6$). (**b**) VEGF-induced mobilization of EPCs (day 4; $n = 5$). (**c**) Laser Doppler analysis of relative blood flow in ischemic limbs 2 weeks after induction of ischemia ($n = 6$). (**d**) Fluorescence *in situ* hybridization analysis of incorporation of bone marrow–derived cells into the vasculature of the limb muscles in female mice transplanted with male bone marrow. (**e**) Representative pictures of wild-type bone marrow–derived Y chromosomes in female wild-type (left) and *Nos3*$^{-/-}$ (right) recipients. Arrows indicate Y chromosomes (green) in incorporated cells. Endothelial cells were identified by immunostaining for CD31 (red).

Nos3$^{-/-}$ mice showed not only profoundly reduced basal activity of pro-MMP-9 (45.5 ± 6.7% compared with wild-type; $P = 0.003$; $n = 4$), but also a blunted increase in pro-MMP-9 activity in response to 5-FU treatment (**Fig. 6c**). Consistent with this observation, incubation of the NO donor *S*-nitrosopenicillamine increased expression of pro-MMP-9 in cultivated bone marrow stromal cells (**Supplementary Fig. 7** online).

Stimulation of MMP-9 by 5-FU leads to cleavage of the membrane-bound Kit ligand (KitL) and subsequent release of soluble KitL[11]. We therefore measured soluble KitL in bone marrow plasma, and found that *Nos3*$^{-/-}$ mice showed a profound impairment of 5-FU-induced release of soluble KitL (**Fig. 6d**). To further delineate the causal contribution of impaired soluble KitL generation to the delayed hematopoietic recovery of *Nos3*$^{-/-}$ mice after 5-FU treatment, we supplemented *Nos3*$^{-/-}$ mice with soluble KitL. For this purpose, *Nos3*$^{-/-}$ mice received twice-daily subcutaneous injections of recombinant mouse soluble KitL, from days 3 to 11 after initial treatment with a single intravenous injection of 5-FU according to a previously published protocol[11]. Soluble KitL treatment rescued the recovery of white blood cell counts and augmented the number of EPCs in *Nos3*$^{-/-}$ mice after 5-FU treatment. The delayed recovery of white blood cells in *Nos3*$^{-/-}$ mice after 14 d of 5-FU treatment (42% inhibition; **Fig. 1c**) was thereby abolished (white blood cell count was 15,144 ± 5,313 cells/μl in *Nos3*$^{-/-}$ mice treated with 5-FU and soluble KitL; $n = 6$; $P < 0.05$ compared with *Nos3*$^{-/-}$ mice treated with 5-FU alone). Notably, white blood cell counts after combined treatment with soluble KitL and 5-FU were higher than the baseline levels. A similar response was previously described in MMP-9-deficient mice[11].

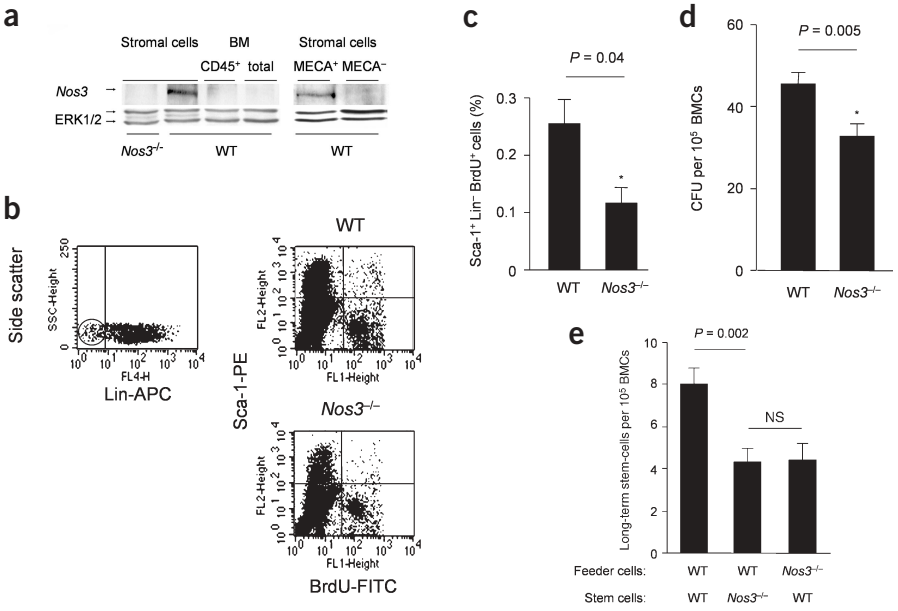

Figure 5 *In vitro* characterization of hematopoietic stem cells from *Nos3*$^{-/-}$ and wild-type (WT) bone marrow. (**a**) Western blot analysis of eNOS in isolated stromal cells, total bone marrow (BM), stromal cell–free (CD45$^+$) bone marrow, and MECA-32$^+$ vascular and MECA-32$^-$ stromal cells. A representative blot is shown ($n = 4$). (**b**) FACS analysis of Lin$^-$ cells (circled) of the lymphocyte-monocyte bone marrow fraction after pulsing with BrdU and staining with BrdU-FITC, Sca-1-PE (phycoerythrin) and a biotinylated lineage cocktail, followed by streptavidin-allophycocyanin (APC). (**c**) Statistical analysis of proliferation (BrdU$^+$) of Sca-1$^+$Lin$^-$ hematopoietic stem cells ($n = 6$). (**d**) Colony-forming capacity of *Nos3*$^{-/-}$ and wild-type total bone marrow cells, as shown by scoring CFUs ($n = 9$). (**e**) Cultivation of long-term stem cells in an *ex vivo* microenvironment produced by stromal cell feeders ($n = 6$).

DISCUSSION

Data from the present study show that eNOS is essential for the functional activity of hematopoietic stem and progenitor cells. Defective mobilization of stem and progenitor cells contributes to the impairment of ischemia-induced neovascularization in *Nos3*$^{-/-}$ mice. These findings are in line with previous studies showing that blockade of endothelial and hematopoietic precursor cell recruitment is sufficient to reduce angiogenesis[8]. The number of bone marrow–derived cells that incorporate into capillary-like structures and express endothelial marker proteins varies in the literature[3,8,23]. In the present study, we found that ~5–7% of capillaries show incorporation of bone marrow–derived cells. Given the large change in capillary density, potential paracrine effects of the infiltrating bone marrow–derived cells are likely to contribute to improved neovascularization, in addition to the cells being incorporated into the

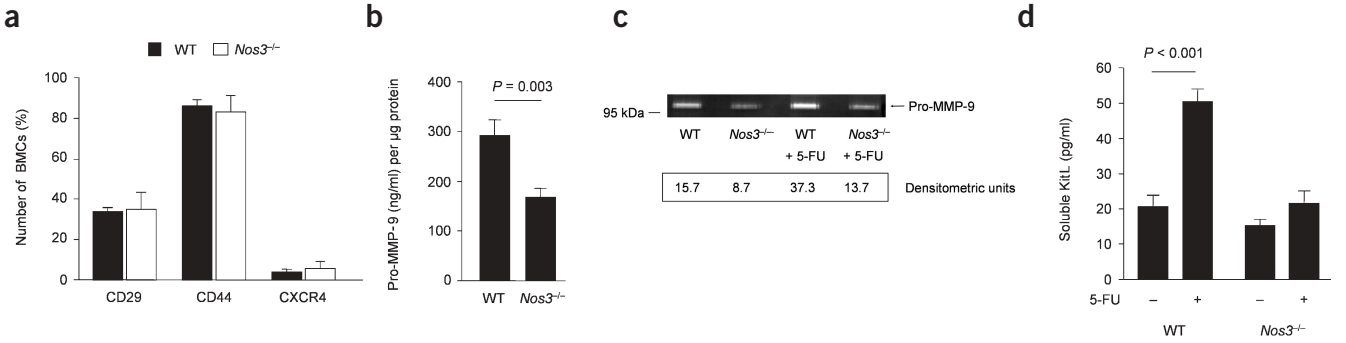

Figure 6 Molecules involved in stem and progenitor cell mobilization. (**a**) Percentage of BMCs in the lymphocyte-monocyte fraction that stained for the integrin CD29, the adhesion molecule CD44 and the chemokine receptor CXCR4, as measured by FACS analysis ($n = 3$–5). (**b**) Expression of pro-MMP-9 in bone marrow plasma of *Nos3*$^{-/-}$ and wild-type (WT) mice, as determined by ELISA ($n = 9$). (**c**) Gelatin zymography of bone marrow plasma before and 3 d after a single 5-FU injection. Densitometric quantification of representative zymography is shown. (**d**) Soluble KitL in bone marrow plasma was measured on day 6 after 5-FU treatment ($n = 6$–8 per group).

endothelial linings. The concept that bone marrow–derived cells can promote neovascularization without being physically incorporated into the endothelial lining has been proposed by a recent report that activation of a suicide gene in bone marrow–derived cells can reduce tumor angiogenesis, although none of the infiltrating cells showed coexpression of endothelial markers in that experimental model[23].

Deficiency in eNOS also reduced hematopoietic recovery and subsequently increased mortality after myelosuppression. A similar phenotype in response to 5-FU and VEGF was recently reported for MMP-9-deficient animals, which show reduced transfer of endothelial and hematopoietic stem cells from quiescent to proliferative niches in the bone marrow[11]. Mice deficient in *Nos3* show not only reduced basal expression and activity of MMP-9, but also a significantly blunted increase in MMP-9 activity in response to 5-FU. Thus, a reduction in MMP-9 may indeed underlie the mobilization defect in *Nos3*$^{-/-}$ mice described in the present study. This hypothesis is supported by recent data identifying MMP-9 as a major target for NO, which activates MMP-9 by *S*-nitrosylation[22]. In addition to regulating MMPs, NO also modulates proliferation, differentiation and apoptosis in various cell types[24–26]. Although our study excludes an effect on stem and progenitor cell apoptosis *in vitro*, we cannot rule out the possibility that endothelium-derived NO interferes with cell cycle progression and/or differentiation of stem cells in the bone marrow. Previous *ex vivo* studies have shown that low concentrations of NO donors, equivalent to those produced by eNOS *in vivo*, can specifically increase the colony-forming activity of white blood cell precursors from CD34$^+$ cells[27].

Our data also indicate that NO in the bone marrow acts primarily in a paracrine manner, as it is generated mainly by vascular cells of the bone marrow stroma. The stromal cell population also contains endothelial cells, and ~30% of the stromal cells express VEGF receptor-2 (Flk-1). Hypoxia-induced upregulation of VEGF activates eNOS predominantly through Flk-1-mediated, Akt-dependent eNOS phosphorylation[28,29]. It is thus possible that VEGF-stimulated NO synthesis in bone marrow stromal cells regulates MMP-9, which in turn induces mobilization of hematopoietic and endothelial progenitor cells[30]. However, increased expression of inducible NOS may also partially compensate for the lack of eNOS, and may thus be responsible for the moderate phenotype observed in *Nos3*$^{-/-}$ mice. Further studies are required to elucidate whether double knockout of the genes encoding eNOS and inducible NOS might create an even more pronounced phenotype.

In addition to the essential role of eNOS in mobilization of stem and progenitor cells from the bone marrow, eNOS is also required for angiogenesis in ischemic tissue. This is indicated by our finding that intravenous infusion of *Nos3*$^{-/-}$ cells or bone marrow engraftment of *Nos3*$^{-/-}$ cells into wild-type animals does not rescue the impaired neovascularization. These data are in accordance with previous studies showing that eNOS is essential for the survival, migration and angiogenic response of mature endothelial cells[24–26]. The definitive mechanism underlying the impaired capacity of *Nos3*$^{-/-}$ to rescue neovascularization is not clear. In our study, *Nos3*$^{-/-}$ cells showed significantly reduced incorporation into ischemic tissue. *Nos3*$^{-/-}$ cells might be more sensitive to apoptosis *in vivo*, leading to a reduction in cell numbers, or might have reduced migratory capacity, resulting in impaired transmigration into the ischemic tissue. However, we did not detect an increase in apoptosis when the *Nos3*$^{-/-}$ bone marrow cells used in the infusion studies were kept *ex vivo* for 24 h, ruling out the possibility that eNOS deficiency directly affects progenitor cell survival.

The results of the present study may have important implications for cardiovascular disease progression. It is well established that endothelial NO bioavailability, which determines the development of coronary artery disease[31], is not only impaired in atherosclerotic coronary arteries, but is also reduced systemically in patients at risk for coronary artery disease[32]. Consequently, endothelial NO synthesis may also be reduced in the bone marrow of heart patients, resulting in reduced mobilization of progenitor cells. Indeed, patients at risk for developing coronary artery disease have significantly reduced numbers of circulating EPCs[33,34]. In addition, reduced numbers of circulating EPCs were recently shown to be associated with endothelial dysfunction in healthy adults[35]. Thus, the defective mobilization of progenitor cells caused by reduced NO bioavailability may impair endogenous vascular repair mechanisms, thereby contributing to the acceleration of vascular aging as well as to reduced neovascularization and cardiac regeneration in patients with ischemic heart disease.

METHODS

Animal experiments. *Nos3*$^{-/-}$ mice and their age- and sex-matched wild-type littermates (C57BL/6J) were purchased from Jackson Laboratories. Human $VEGF_{165}$ (500 μg per kg per d) or saline were subcutaneously administered once a day for a period of 3 d, along with 5-FU in a single dose of 250 mg/kg. Recombinant mouse soluble KitL was injected twice daily (150 μg/kg).

Bone marrow transplantation. CD45$^+$ bone marrow cells were isolated using CD45-bound microbeads and an automated separation device (Miltenyi Biotec). Recipient mice were lethally irradiated with 9.5 Gy and received an intravenous injection of 10^6 donor bone marrow cells 24 h after irradiation. As a control for transplantation efficiency, bone marrow from CD45.1 mice was transplanted into *Nos3*$^{-/-}$ and wild-type mice (both CD45.2; **Supplementary Fig. 8** online)[36]. Hind-limb ischemia was induced 6 weeks after bone marrow transplantation.

Hind-limb ischemia. The proximal femoral artery, including the superficial and deep branches, and the distal portion of the saphenous artery were ligated in the right hind limb[37]. Two weeks later, we measured blood flow ratios in the ischemic (right) versus normal (left) limbs, using a laser Doppler blood flow imager (Moor Instruments; see **Supplementary Fig. 9** online for time course). Capillary density in the semimembraneous and adductor muscles was determined in 5-μm cryosections using FITC-conjugated monoclonal antibody to CD31 (BD PharMingen). The endothelial phenotype was confirmed by immunostaining for the pan-endothelial marker MECA-32 (BD PharMingen). Injected cells were labeled with CellTracker CM-Dil (Molecular Probes). Bone marrow–derived cells were identified by fluorescence *in situ* hybridization[38] (probe from Cambio).

BrdU proliferation assay. Bone marrow was collected aseptically by flushing tibias and femurs, and was pulsed with 10 μM BrdU (BrdU flow kit, BD PharMingen) for 1 h before immunostaining for surface antigens. Cells were incubated with phycoerythrin-conjugated rat antibody to mouse Sca-1, and a cocktail of biotinylated antibodies to the lineage markers CD3, B220, CD11b, Gr-1 and TER119 (BD PharMingen) for 20 min at 4 °C, followed by streptavidin-allophycocyanin (BD PharMingen). Cells were fixed and permeabilized with Cytofix/Cytoperm buffer (BD PharMingen) according to the manufacturer's instructions, stained with FITC-conjugated antibody to BrdU (BrdU flow kit, BD PharMingen) and fixed in 1% formaldehyde and PBS. The analysis was done using a FACSCalibur with CellQuest software (BD PharMingen).

Colony-forming-unit assay. Total bone marrow cells (10^5 per well) were plated in 1.5 ml of 1% methylcellulose (Methocult, StemCell Technologies) containing 15% FBS, 1% BSA, 10 μg/ml human insulin, 200 μg/ml human transferrin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml mouse stem cell factor, 50 ng/ml human VEGF, 10 ng/ml mouse interleukin-3, 10 ng/ml human interleukin-6 and 3 U/ml human erythropoietin. Colonies (>50 cells) were scored on day 7.

Stromal cell culture. Adherent cells from total bone marrow were cultured in IMDM with 10% FCS, 50 μM 2-mercaptoethanol and antibiotics for 6 weeks, until the formation of 80% confluent monolayers. MECA-32-positive cells were selected using magnetic beads (antibody from BD PharMingen).

Long-term stem cell assay. Bone marrow stromal layers were irradiated (20 Gy), overlaid with 10^6 wild-type or *Nos3*$^{-/-}$ total bone marrow cells and cultured in IMDM supplemented with 10% FCS, 10% horse serum, 10^{-5} M hydrocortisone (Sigma) and antibiotics in 12-well plates. Cells were fed twice a week with IMDM. After 35 d, nonadherent cells were flushed from the stromal feeder layers, 10^5 cells were plated in the methylcellulose clonal assay and colonies were scored after 7 d.

EPC assay. For EPC culture, mononuclear cells (4×10^6 cells/well) were isolated from spleen-cell homogenates as previously described[39], in a modified protocol using 100 ng/ml VEGF (Peprotech) in the culture medium. The identity of EPCs was confirmed by Dil-labeled acetylated low-density lipoprotein/lectin staining and Flk-1 expression.

Detection of MMP-9 and soluble KitL. For gelatin zymography, bone marrow plasma was collected by flushing femurs and tibias with a total of 0.5 ml PBS. After spinning, supernatants were analyzed for metalloproteinase activity by gelatinolytic zymography as described[40]. Mouse pro-MMP-9 (20 μg protein) and soluble KitL expression were measured by ELISA (R&D Systems).

Western blot analysis. Cells were lysed and western blot analysis was done as described[28], using monoclonal antibody to eNOS (BD Biosciences) followed by mouse antibody to horseradish peroxidase (Santa Cruz Biotechnology) and reprobing for ERK1/2 (Cell Signaling Technology).

Statistical analysis. Comparisons between groups were analyzed by two-sided *t*-test or ANOVA for experiments with more than two subgroups. *Post hoc* range tests and pairwise multiple comparisons were done using the two-sided *t*-test with Bonferroni adjustment. The Kaplan-Meier method was used to construct survival curves, and the probability of survival between pairs was compared with a log rank test. $P < 0.05$ was considered statistically significant. All analyses were done with SPSS 11.5 software.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGEMENTS

We thank M. Muhly-Reinholz, S. Rhiel, T. Röxe and A. Knau for their excellent technical assistance, and H.E. Schaefer (University of Freiburg) for helpful discussion. This work was supported by the Sonderforschungsbereich (SFB 553) and the Alfried Krupp-Stiftung to S.D.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 24 July; accepted 22 September 2003
Published online at http://www.nature.com/naturemedicine/

1. Isner, J.M. & Losordo, D.W. Therapeutic angiogenesis for heart failure. *Nat. Med.* **5**, 491–492 (1999).
2. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1**, 27–31 (1995).
3. Asahara, T. *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964–967 (1997).
4. Takahashi, T. *et al.* Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat. Med.* **5**, 434–438 (1999).
5. Shi, Q. *et al.* Evidence for circulating bone marrow-derived endothelial cells. *Blood* **92**, 362–367 (1998).
6. Risau, W. Mechanisms of angiogenesis. *Nature* **386**, 671–674 (1997).
7. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* **6**, 389–395 (2000).
8. Lyden, D. *et al.* Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Med.* **7**, 1194–1201 (2001).
9. Shintani, S. *et al.* Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation* **103**, 897–903 (2001).
10. Moore, M.A. *et al.* Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. *Ann. NY Acad. Sci.* **938**, 36–45 (2001).
11. Heissig, B. *et al.* Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* **109**, 625–637 (2002).
12. Asahara, T. *et al.* VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* **18**, 3964–3972 (1999).
13. Morrison, S.J., Shah, N.M. & Anderson, D.J. Regulatory mechanisms in stem cell biology. *Cell* **88**, 287–298 (1997).
14. Kiger, A.A., White-Cooper, H. & Fuller, M.T. Somatic support cells restrict germline stem cell self-renewal and promote differentiation. *Nature* **407**, 750–754 (2000).
15. Lapidot, T. & Petit, I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp. Hematol.* **30**, 973–981 (2002).
16. Murohara, T. *et al.* Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.* **101**, 2567–2578 (1998).
17. Taylor, D.A., Hruban, R., Rodriguez, E.R. & Goldschmidt-Clermont, P.J. Cardiac chimerism as a mechanism for self-repair: does it happen and if so to what degree? *Circulation* **106**, 2–4 (2002).
18. Prosper, F., Stroncek, D., McCarthy, J.B. & Verfaillie, C.M. Mobilization and homing of peripheral blood progenitors is related to reversible downregulation of α4β1 integrin expression and function. *J. Clin. Invest.* **101**, 2456–2467 (1998).
19. Vermeulen, M. *et al.* Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. *Blood* **92**, 894–900 (1998).
20. Peled, A. *et al.* Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* **283**, 845–848 (1999).
21. Hattori, K. *et al.* Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat. Med.* **8**, 841–849 (2002).
22. Gu, Z. *et al.* S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* **297**, 1186–1190 (2002).
23. De Palma, M., Venneri, M.A., Roca, C. & Naldini, L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat. Med.* **9**, 789–795 (2003).
24. Ziche, M. *et al.* Nitric oxide mediates angiogenesis *in vivo* and endothelial cell growth and migration *in vitro* promoted by substance P. *J. Clin. Invest.* **94**, 2036–2044 (1994).
25. Murohara, T. *et al.* Role of endothelial nitric oxide synthase in endothelial cell migration. *Arterioscler. Thromb. Vasc. Biol.* **19**, 1156–1161 (1999).
26. Dimmeler, S., Haendeler, J., Nehls, M. & Zeiher, A.M. Suppression of apoptosis by nitric oxide via inhibition of ICE-like and CPP32-like proteases. *J. Exp. Med.* **185**, 601–608 (1997).
27. Shami, P.J. & Weinberg, J.B. Differential effects of nitric oxide on erythroid and myeloid colony growth from CD34$^+$ human bone marrow cells. *Blood* **87**, 977–982 (1996).
28. Dimmeler, S. *et al.* Activation of nitric oxide synthase in endothelial cells via Akt-dependent phosphorylation. *Nature* **399**, 601–605 (1999).
29. Fulton, D. *et al.* Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597–601 (1999).
30. Eriksson, U. & Alitalo, K. VEGF receptor 1 stimulates stem-cell recruitment and new hope for angiogenesis therapies. *Nat. Med.* **8**, 775–777 (2002).
31. Schachinger, V., Britten, M.B. & Zeiher, A.M. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* **101**, 1899–1906 (2000).
32. Zeiher, A.M. Endothelial vasodilator dysfunction: pathogenetic link to myocardial ischaemia or epiphenomenon? *Lancet* **348**, S10–S12 (1996).
33. Vasa, M. *et al.* Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ. Res.* **89**, E1–E7 (2001).
34. Tepper, O.M. *et al.* Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* **106**, 2781–2786 (2002).
35. Hill, J.M. *et al.* Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N. Engl. J. Med.* **348**, 593–600 (2003).
36. Fiering, S.N. *et al.* Improved FACS-Gal: flow cytometric analysis and sorting of viable eukaryotic cells expressing reporter gene constructs. *Cytometry* **12**, 291–301 (1991).
37. Heeschen, C. *et al.* Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nat. Med.* **7**, 833–839 (2001).
38. Mezey, E. *et al.* Transplanted bone marrow generates new neurons in human brains. *Proc. Natl. Acad. Sci. USA* **100**, 1364–1369 (2003).
39. Dimmeler, S. *et al.* HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J. Clin. Invest.* **108**, 391–397 (2001).
40. Rajagopalan, S., Meng, X.P., Ramasamy, S., Harrison, D.G. & Galis, Z.S. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases *in vitro*. Implications for atherosclerotic plaque stability. *J. Clin. Invest.* **98**, 2572–2579 (1996).

ERRATUM: Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells

Alexandra Aicher, Christopher Heeschen, Christiane Mildner-Rihm, Carmen Urbich, Christian Ihling, Katja Technau-Ihling, Andreas M Zeiher & Stephanie Dimmeler

Nature Med. **9, 1370–1376 (2003)**

In the original version of this article, Fig. 2b was incorrect. The correct panel appears below.